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ORIGINAL PAPER

Transcriptional activity of *Pseudomonas aeruginosa fhp* promoter is dependent on two regulators in addition to FhpR

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Abstract The regulation of flavohemoglobin expression is complex and depending on its host organism requires a wide variety of different transcriptional regulators. In *Pseudomonas aeruginosa*, the flavohemoglobin (Fhp) and its cognate regulator FhpR form an NO-sensing and detoxifying system regulated by their common bidirectional promoter P_{fhp}/P_{fhpR}. The intergenic *fhp–fhpR* region of *P. aeruginosa* PAO1 was used as a bait to isolate proteins affecting the transcription of *fhp* and *fhpR*. In addition to the FhpR, we identified two previously uncharacterized *P. aeruginosa* proteins, PA0779 and PA3697. Both PA0779 and PA3697 were found to be essential for NO_3^- and NO_2^- induced P_{fhp} activity under aerobic and low-oxygen conditions, and needed for the full function of P_{fhp}/P_{fhpR} as NO responsive regulatory circuit under aerobic conditions. In addition, we show that the transcriptional activity of P_{fhpR} is highly inducible upon addition of SNP under aerobic conditions, but not by NO_3^-, NO_2^- or under low-oxygen conditions, supporting the findings that FhpR is not the only factor affecting flavohemoglobin expression in *P. aeruginosa*.

Keywords *P. aeruginosa* · Flavohemoglobin · *fhp* · Transcriptional regulation · *fhpR* · PA0779 · PA3697

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Abbreviations

CF	Cystic fibrosis
Fhp	*P. aeruginosa* flavohemoglobin
FhpR	Transcriptional activator of *P. aeruginosa* flavohemoglobin
FlavoHb	Flavohemoglobin
HmpEc	*E. coli* flavohemoglobin
HmpStm	*S. enterica* serovar typhimurium flavohemoglobin
LB	Luria Bertani
NAR	Nitrate reductase
NIR	Nitrite reductase
NOR	Nitric oxide reductase
NO	Nitrogen monoxide/nitric oxide
NorR	Transcriptional activator of *E. coli* flavorubredoxin oxidoreductase
NorVW	*E. coli* flavorubredoxin oxidoreductase
OD	Optical density
P_{fhp}	Promoter of *P. aeruginosa* flavohemoglobin
P_{fhpR}	Promoter of *P. aeruginosa* transcriptional activator FhpR
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
TE	Tris–EDTA
TGED	Tris–Glycerol–EDTA–DTT

Introduction

Flavohemoglobins (flavoHbs) are important NO-detoxifying proteins of many microorganisms. Under aerobic or microaerobic conditions flavoHbs possess dioxygenase activity, which enables them to convert NO to NO_3^- (Gardner et al. 1998; Hausladen et al. 1998, 2001), a reaction which

has been postulated to be the primary reaction of flavoHbs. Under anaerobic conditions, they have been shown to reduce NO to N_2O, but this reaction is much slower than in the presence of oxygen (Hausladen et al. 1998, 2001; Kim et al. 1999; Poole 2005). There exists evidence that flavoHbs have an important role in protecting pathogenic bacteria against NO. In *Salmonella enterica* serovar Typhimurium, FlavoHb (HmpStm) was able to protect the bacterium from macrophage related NO-killing (Stevanin et al. 2002), and recently, HmpStm was also shown to promote *Salmonella* virulence during chronic and acute lethal infection of mice (Bang et al. 2006).

Pseudomonas aeruginosa is an opportunistic human pathogen causing serious infections in individuals with impaired host defense, especially in cystic fibrosis (CF) patients (Pollack 2000). The expression of flavohemoglobin (Fhp) has been suggested to be advantageous for *P. aeruginosa* survival in the CF lung (Firoved and Deretic 2003), which is rich in NO_3^- and NO_2^-, and low in oxygen (Jones et al. 2000). NO_3^- and NO_2^- are most likely produced via oxidation of NO, which is formed in airway epithelia. NO is expected to be converted to NO_2^- via autoxidation and oxidation reactions, which are catalyzed by metalloproteins. NO_2^- is possibly oxidized further to NO_3^- by peroxidases, using neutrophil-generated H_2O_2 as an oxidant (reviewed by Hassett et al. 2002). In addition, $ONOO^-$ is formed when NO reacts directly with neutrophil-derived superoxide (O_2^-) (Huie and Padmaja 1993). In a mucoid supressor strain of *P. aeruginosa* PAO1 (*mucA22 sup-2*), isolated frequently from the lungs of CF patients, *fhp* expression was increased several fold compared to the wild-type strain, suggesting that *fhp* is upregulated to compensate for the reduced protection against reactive nitrogen (RNS) and oxygen species (ROS) normally provided by the mucoid alginate producing phenotype (Firoved and Deretic 2003). Recently, the physiological role of *P. aeruginosa* flavohemoglobin was suggested to be detoxification of NO under aerobic conditions, but it was also shown to be highly upregulated under microaerobic and anaerobic conditions upon addition of NO gas, sodium nitroprusside (SNP) *s*-nitrosoglutathione (GSNO), NO_3^- and NO_2^- (Arai et al. 2005). This suggests that as other flavoHbs such as from *E. coli* and *Salmonella*, *P. aeruginosa* Fhp also possesses NO reducing activity converting NO to N_2O under anaerobic conditions.

The regulation of *flavoHb* expression in bacteria is complex. Several transcription factors such as CRP/FNR-like proteins, two-component systems, NO-responsive activators and nitrite-sensitive repressors, are predicted to control *flavoHb* expression (Frey and Kallio 2003; Rodionov et al. 2005; Koskenkorva et al. 2006). Recently, it was shown that NO induces *hmpStm* promoter activity by reversibly inactivating the NsrR repressor of *S. typhimurium* (Bang et al. 2006), and that paraquat induced HmpStm activity is activated directly or indirectly by RamA (Hernández-Urzúa et al. 2007). *Bacillus subtilis hmp* (*hmpBs*), is strongly induced during oxygen limited conditions, and induction is dependent on the two-component regulatory pair ResD-ResE, and derepression by NsrR (Nakano et al. 2006).

In *P. aeruginosa*, the transcription of the *fhp* promoter (P_{fhp}) is σ^{54}-dependent. The transcription of P_{fhp} is activated by FhpR, which is a NorR type σ^{54}-dependent activator having three binding sites within P_{fhp} (Büsch et al. 2004; Arai et al. 2005). The genes encoding Fhp and FhpR are juxtaposed, but divergently oriented, and share their promoter regions (Büsch et al. 2004; Arai et al. 2005; Rodionov et al. 2005; Hernández-Urzúa et al. 2007). Thus, Fhp and FhpR form an NO-sensing and detoxifying system regulated by their common bidirectional promoter P_{fhp}/P_{fhpR}. The possible involvement of other factors than FhpR in the regulation of P_{fhp}/P_{fhpR} under microaerobic and anaerobic conditions has remained so far elusive. In this study, the *fhp-fhpR* intergenic region DNA of *P. aeruginosa* PAO1 was used as a bait to isolate proteins from the soluble total protein fraction in vitro. In addition to the FhpR, we identified two previously uncharacterized *P. aeruginosa* proteins PA0779 and PA3697, both of which were found to be essential for NO_3^- and NO_2^- induced P_{fhp} activity under aerobic and low-oxygen conditions, and needed for the full function of P_{fhp}/P_{fhpR} as NO responsive regulatory circuit under aerobic conditions.

Materials and methods

Bacterial strains, plasmids and selection media

The origins and characteristics of bacterial strains and plasmids are listed in Table 1. Genomic DNA from *P. aeruginosa* PAO1 was used to obtain the *fhp–fhpR* intergenic sequence. *E. coli* SM10 (Simon et al. 1983) was used for cloning and plasmid propagation. *P. aeruginosa* PAO1 wild-type (wt) and strains 15 F1 (PA0779 mutant) and 19 D10 (PA3697 mutant) (Lewenza et al. 2005) were used to analyze the transcriptional activity of P_{fhp} and P_{fhpR}. *E. coli* was grown at 37°C in Luria–Bertani (LB) medium or on LB agar (Sambrook and Russell 2001). *P. aeruginosa* strains were grown in LB medium and on *Pseudomonas* isolation agar (PIA; Becton Dickinson, Basel, Switzerland), if not otherwise stated. For plasmid selection, tetracycline was added to the growth media: 10 μg/ml for *E. coli* SM10, 200 μg/ml for *P. aeruginosa* PAO1, and 400 μg/ml for *P. aeruginosa* PA0779 and PA3697 mutants. Overnight cultures were grown in 5 ml LB. All cultivations were performed at 37°C and 250 rpm on an orbital shaker with a shaking diameter of 5 cm (Kühner AG, Basel, Switzerland).

Table 1 Strains and plasmids

Strain or plasmid	Characteristics	Reference
P. aeruginosa		
PAO1	ATCC 15692, wild-type	
15 F1	PA0779::*lux*	Lewenza et al. (2005)
19 D10	PA3697::*lux*	Lewenza et al. (2005)
TK1	P_{fhp}-*lacZ* integrated at the *attP* site of PAO1	This work
TK2	P_{fhpR}-*lacZ* integrated at the *attP* site of PAO1	This work
TK3	P_{fhp}-*lacZ* integrated at the *attP* site of 15 F1	This work
TK4	P_{fhpR}-*lacZ* integrated at the *attP* site of 15 F1	This work
TK5	P_{fhp}-*lacZ* integrated at the *attP* site of 19 D10	This work
TK6	P_{fhpR}-*lacZ* integrated at the *attP* site of 19 D10	This work
E. coli SM10	Km^r, *thi*-1, *thr*, *leu*, *tonA*, *lacY*, *supE*, *recA*::RP4–2-Tc::Mu, λpir	Simon et al. (1983)
Plasmids		
mini-CTX-*lacZ*	Tc^r, integation proficient *lacZ* transcriptional fusion vector	Becher and Schweizer (2000)
P_{fhp}-*lacZ*	Tc^r, *fhp::lacZ* transcriptional fusion	This work
P_{fhpR}-*lacZ*	Tc^r, *fhpR::lacZ* transcriptional fusion	This work

All chemicals were obtained from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland).

Motility assays

To test swimming motility, bacteria from colonies grown overnight at 37°C on LB agar (1.5% w/v) were inoculated with a sterile toothpick on 0.3% LB agar. Plates were wrapped in Parafilm to prevent evaporation and incubated overnight at 30°C. Swimming was measured as the diameter of the motility zone after 18 h of incubation (Köhler et al. 2000).

To test the swarming motility, strains grown overnight at 30°C on swimming agar plates were inoculated on swarming agar plates (8.5 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl and 5 g agar per liter, supplemented with 1 mM $MgSO_4$, 0.2% glucose and 0.5% casamino acids). Plates were incubated at 30°C overnight and swarming motility was assessed as the branching growth of the strain from the inoculation point over the agar surface (Köhler et al. 2000).

Twitching motility was tested on 1% LB agar containing 0.05% 2,3,5-triphenyltetrazolium chloride. Strains grown overnight on LB agar (1.5% w/v) were stab inoculated with a sharp and sterile toothpick to the bottom of a Petri dish. Plates were incubated for 48 h at 37°C, and the diameter of the twitching motility was measured (Semmler et al. 1999).

Molecular biology methods

DNA manipulations were performed according to standard procedures (Sambrook and Russell 2001). Plasmid isolations were done using Spin Miniprep Kit (Qiagen, Basel, Switzerland). PAO1 chromosomal DNA, isolated with DNeasy Tissue Kit (Qiagen), was used in all PCR amplification reactions as a template. PCR reactions were performed in a Techne Unit TC-412 (Techne Inc., UK) using *Taq* DNA-polymerase (Fermentas, Nunningen, Switzerland). Oligonucleotides were obtained from Microsynth (Balgach, Switzerland). Restriction enzymes were purchased from New England Biolabs (Ipswich, MA) and Fermentas. PCR fragments to be coupled with Dynabeads were purified using QIAquick PCR purification Kit (Qiagen, Switzerland). DNA fragments for cloning were extracted from agarose gels using Spin Miniprep Kit (Qiagen).

Construction of DNA–Dynabead affinity complexes

A 354 bp DNA fragment (bait) containing the 154 bp long *fhp–fhpR* intergenic region and the 5′-end of the *fhp* structural gene (200 bp) was PCR amplified using PaPFwdDyna1 and biotinylated reverse primer PaRevBiotin (Table 2). A 200 bp DNA fragment (control) containing only the 5′-end of the *fhp* structural gene was amplified using PaFwdDyna3 and PaRevBiotin primers (Table 2) to be used as a negative control. Binding of the biotinylated bait and control DNA to the Magnetic Dynabeads M-280 Streptavidin (Dynal Biotech ASA, Norway) was done following the manufacturers instructions. 120 pmol of bait (27,720 ng) and control (15,840 ng) DNA was coupled to Dynabeads using 4.2 and 2.4 mg of beads, respectively, in 5 g/ml final concentration of beads in the binding buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1.0 M NaCl), at room temperature for 15 min with periodical mixing. Formed DNA–Dynabead complexes were washed once with the binding buffer, and stored in 100 µl of TE buffer (Sambrook and Russell 2001) at 4°C.

Table 2 Oligonucleotides used for amplifying promoter DNA to be coupled with Dynabeads, and for cloning

Primer	Sequence (5′–3′)	Restriction site (underlined in sequence)	Use
PaPFwdDyna1	TTCGACCTCTTTGCAGTCCTAATGACTC	–	Amplifying bait DNA for Dynabeads
PaPFwdDyna3	CTCATCAAAGCCACCGTACCGCT	–	Amplifying control DNA for Dynabeads
PaRevBiotin	BIOTIN-C_{12}-GATGTGCCTGGCGTACATCAGCA	–	Amplifying bait and control DNA for Dynabeads
P_{hmp}-fwd	CCCAAGCTTGTAGGGATCGGGCAGGCCG	*Hind*III	Cloning of P_{fhp} promoter
P_{hmp}-rev	GCGGATCCACATGCTTGTTCACCACCTTGG	*Bam*HI	Cloning of P_{fhp} promoter
P_{norR}-fwd	CCCAAGCTTACATGCTTGTTCACCACCTTGG	*Hind*III	Cloning of P_{fhpR} promoter
P_{norR}-rev	GCGGATCCTAGGGATCGGGCAGGCCGC	*Bam*HI	Cloning of P_{fhpR} promoter
Pser-up	CGAGTGGTTTAAGGCAACGGTCTTGA	–	Screening integration at *attP* site within PAO1
LacZ-SF	GGAGCCCGTCAGTATCGGCGGAG	–	Screening integration at *attP* site within PAO1

Isolation of proteins

LB (1,950 ml) media was inoculated with 50 ml of *P. aeruginosa* PAO1 culture grown overnight. Two consecutive batch cultivations with a total volume of 2.7 l were performed in a KLF 2000 bioreactor (Bioengineering AG, Wald, Switzerland) at 37°C, with 1,200 rpm and 1 vvm aeration rate. Cells were harvested at late exponential growth phase at 2,600 × *g* and 4°C for 15 min. Cells were washed once with PBS, centrifuged as above and resuspended in TGED buffer (20 mM Tris–HCl, pH 8; 10% glycerol; 1 mM EDTA; 1 mM DDT) containing 100 mM NaCl. Phenyl methyl sulfonyl fluoride (PMSF) was added to 1 mM final concentration, and cells were broken by Aminco French Pressure Cell (SLM Instruments, Inc., Urbana, IL) at 6,892.7 × 10^3 Pa. The cell debris was removed by ultracentrifugation at 247,400 × *g* and 4°C for 1 h.

DNA–Dynabead complexes in 100 µl TE buffer were centrifuged and TE buffer was removed. Beads were resuspended in 500 µl TGED buffer, which was mixed with 30 ml of soluble protein fraction containing 100-fold excess of salmon sperm DNA (relative to the DNA coupled to Dynabeads in nanograms) and incubated with gentle shaking at room temperature for 3 h. Total protein fraction was removed, and protein–DNA–Dynabead complexes were washed two times with 100–200 µl TGED buffer containing 100 mM NaCl and fivefold excess salmon sperm DNA, and twice with 100–200 µl TGED buffer with 200 mM NaCl. Proteins were eluted from the DNA–Dynabead complexes by incubating the beads for 15 min on ice in 100 µl TGED buffer containing 1 M NaCl. Protein eluates were desalted by drop dialysis with VS type membranes (0.025 µm mean pore size, Millipore, USA) for 30 min, and concentrated to approximately 8 µl in a Hetovac VR-1 (High Technology of Scandinavia, Denmark). Eluates were run on a 12.5% SDS-PAGE (Laemmli 1970). Protein bands were visualized using GelCode Blue stain reagent (Pierce, USA), excised, and in-gel trypsin digested according to a protocol provided at the Website of York University (http://www.biol.yorku.ca/cm/proteomics/). Matrix Assisted Laser Desorption and Ionization Time of Flight Mass Spectrometer (MALDI-TOF-MS; Voyager Elite; PerSeptive Biosystems Inc., USA) was used for peptide mass fingerprinting, and the obtained data was analyzed using the Mascot Distiller program (Matrix Science Inc., USA).

Construction of *lacZ* reporter strains

Plasmid P_{fhp}-*lacZ* was constructed by PCR amplifying a 0.7-kb fragment from PAO1 genomic DNA using P_{hmp}-fwd and P_{hmp}-rev, and plasmid P_{fhpR}-*lacZ* by using P_{norR}-fwd and P_{norR}-rev (Table 2). The amplified fragments were digested with *Hind*III and *Bam*HI and inserted into the corresponding sites of mini-CTX-*lacZ* (Becher and Schweizer 2000). P_{fhp}-*lacZ* and P_{fhpR}-*lacZ* were transformed into *E. coli* SM10 (Simon et al. 1983) and propagated into *P. aeruginosa* PAO1, 15 F1 and 19 D10 strains (Table 1) by conjugation as described elsewhere (Hoang et al. 2000). Integration at the *attP* site in *P. aeruginosa* genome was confirmed by colony PCR (Frey et al. 2000) using Pser-up and LacZ-SF (Table 2).

Cultivations with *lacZ* reporter strains

The transcriptional activities of P_{fhp} and P_{fhpR} were studied with cultivations in 96-deep well plates (Polylabo, Geneva, Switzerland) under aerobic and low-oxygen conditions. M9 minimal medium (Fuhrer et al. 2005) without the vitamin solution, and modified synthetic medium (Wood 1978; Arai et al. 2005) were used for aerobic and low-oxygen conditions, respectively. Low-oxygen conditions were achieved by placing the cultivation vessels in airtight containers,

which were then flushed with nitrogen gas, and sealed. All cultures were incubated at 37°C and 250 rpm on an orbital shaker with a shaking diameter of 5 cm (Kühner AG, Basel, Switzerland).

Overnight cultures of *P. aeruginosa* reporter strains were grown in 5 ml LB and diluted 1/10 with LB to give 5 ml precultures, which were grown for one hour. For 96-deep well plate cultures, 1,000 µl of medium/well was inoculated with 50 µl of optical density (OD_{600}) adjusted (OD_{600} = 1) preculture to a starting OD_{600} of 0.025. For low-oxygen cultivations 15 µl of reactive nitrogen species (RNS) were supplemented to the medium to a final concentration of 80 mM of $NaNO_3$, 20 mM of $NaNO_2$, 3 mM of SNP, or 1 mM of peroxynitrite ($ONOO^-$) at the point of inoculation. Peroxynitrite was prepared as described previously (Koppenol et al. 1996). Cultivation vessels were placed in airtight containers, which were flushed with nitrogen gas for 3 min, and sealed. Fifty microliters of samples were withdrawn after 16 h of cultivation for immediate OD_{600} measurement and β-galactosidase activity assay. For aerobic cultivations, RNSs were added after 3.5 h of cultivation, and 50 µl samples were withdrawn at 0.5 h after RNS supplementation.

β-galactosidase assay

Promoter activity was measured with a direct kinetic assay of β-galactosidase (β-gal) activity in 96-well format. Fifty microliters of sample (diluted with PBS, if necessary) was incubated for 5 min with 40 µl of Z-buffer (Miller 1972) and 10 µl of lysis mixture (Putnam and Koch 1975). Twenty microliters of of *o*-nitrophenyl-β-D-galactoside (ONPG, 4 mg/ml in Z-buffer) was added, and absorbance was measured at 420 nm. β-gal activity is reported in Miller Units (U) (Miller 1972).

In vivo activities of denitrification enzymes

PAO1, 15 F1 and 19 D10 were grown in LB medium supplemented with 20 mM $NaNO_3$ in a sealed vial at 37 °C until stationary phase. The air in the vial was replaced with argon after inoculation.

In vivo nitrate reductase (NAR) and nitrite reductase (NIR) activities were determined as described previously (Arai et al. 1997). Cells were suspended in 50 mM potassium phosphate buffer (pH 7.5). The reaction mixture was composed of 50 mM potassium phosphate (pH 7.5), 50 mM sodium lactate, and appropriate amount of cell suspension. The reaction was performed under an argon atmosphere in a sealed vial. The reaction was started by addition of 1 mM $NaNO_3$ or 1 mM $NaNO_2$ to the reaction mixture. The activity was determined by measuring the residual substrate concentration after incubation for 60 min at 37°C. Nitrate concentration was determined by brucine method. Nitrite concentration was determined by diazotization and coupling reactions with sulphanilic acid and naphthylethylenediamine (Nicholas and Nason 1957).

In vivo nitric oxide reductase (NOR) activity (anaerobic nitric oxide consumption activity) was determined as described previously by using Apollo 4000 free radical analyzer equipped with a 2-mm ISO-NOP NO electrode and an ISO-OXY-2 O_2 electrode (WPI, Sarasota, FL) (Arai et al. 2005). Cells were suspended in 50 mM sodium phosphate buffer (pH 7.0). The reaction was performed in a multiport measurement chamber (WPI) containing 2 ml of reaction buffer [50 mM NaH_2PO_4, 7.6 mM $(NH_4)_2SO_4$, 1.7 mM sodium citrate, and 10 mM glucose] at 37°C. Headspace gas of the chamber was removed by fitting a stopper. Oxygen in the reaction mixture was removed by incubation for some minutes with 2 units/ml of glucose oxidase and 130 units/ml of catalase. Two microliters of NO-saturated water (final NO concentration ~2 µM) and 40 µl of cell suspension was added subsequently to the reaction. The electrode signal was monitored and the in vivo NOR activity was calculated from the difference of NO consumption rates before and after the addition of the cell suspension to the reaction mixture. The protein concentration of the cell suspension was determined by the Bradford method using the protein assay kit of Bio-Rad (Hercules, CA) after the cells were lysed by boiling with 0.33 N NaOH. Bovine serum albumin was used as a standard.

Results

Isolation and identification of proteins

We used biotinylated *fhp–fhpR* intergenic region DNA, coupled to streptavidin coated magnetic beads, to isolate proteins, which possibly have an effect on the transcription of P_{fhp} and/or P_{fhpR}. Six protein bands were reproducibly detected on the SDS-PAGE gel, in the lane for eluate from the bait DNA (Fig. 1, lane b), but some of them were very weak (only bands 1, 2, 4, and 5 are clearly visible in Fig. 1). No proteins bands were visible in the lane for eluate from the control DNA (Fig. 1, lane c). Thus, all proteins eluted from the bait were binding specifically to the *fhp–fhpR* intergenic region or via protein–protein interaction to each other, since the control DNA contained only the 5′-end of the *fhp* structural gene. This result suggests the involvement of the isolated proteins in the transcriptional machinery of P_{fhp}/P_{fhpR}. Six protein bands (Fig. 1, bands 1–6) were excised from the gel and in-gel trypsin digested. Using MALDI-TOF-MS, peptide fingerprints of five proteins could be reproducibly identified (Table 3). The 37 and 151 kDa proteins were identified as the α and β chains of

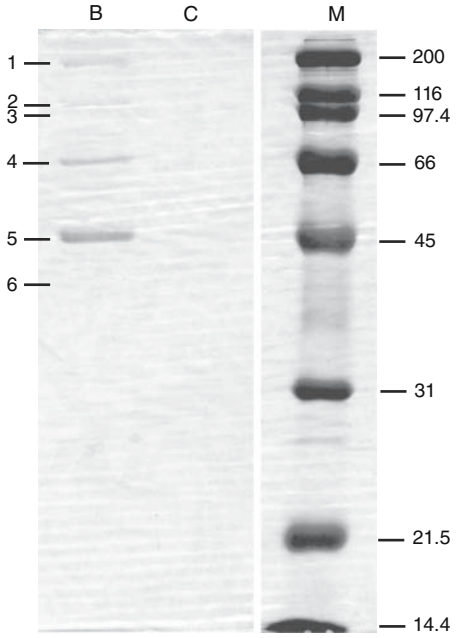

Fig. 1 Proteins binding to bait (**b**) and control (**c**) DNA separated on 12.5% SDS-PAGE gel. Eight proteins (1, 2, 4 and 5 can be seen on the picture) were eluted from the bait DNA, which consisted of 157 bp of the intergenic region and 200 bp of the *fhp* gene 5′-end. Eluate from control DNA, which consisted of the 200 bp of the *fhp* gene 5′-end, contained no proteins. Bands 1–6 were isolated from the gel, in-gel trypsin digested and subjected to MALDI-TOF-MS analysis. M stands for molecular weight marker

Table 3 MALDI-TOF-MS identified proteins binding to the P_{fhp}/P_{fhpR} region in vitro

Protein band	Size (kDa)	PA number	Description
1	151.3	PA4270	DNA-directed RNA polymerase β chain
2	88.6	PA0779	ATP-dependent protease La
3	~ 80.0	–	No match
4	57.7	PA2665	FhpR
5	48.8	PA3697	Conserved hypothetical protein
6	36.7	PA4238	DNA-directed RNA polymerase α chain

P. aeruginosa DNA-directed RNA polymerase. The 57.5 kDa protein was identified to be FhpR, which has previously been shown to have three putative binding sites within the P_{fhp} (Büsch et al. 2004), and which is known to activate RNS induced P_{fhp} transcription (Arai et al. 2005). More interestingly, two previously uncharacterized proteins of 88.6 and 48.8 kDa were identified as PA0779 and PA3697, respectively.

PA0779 has a typical ATP-binding region A signature, a Lon protease (S16) C-terminal proteolytic domain, and an N-terminal ATP-dependent protease La (LON) domain (Marchler-Bauer and Bryant 2004). It shows 61% similarity to human mitochondrial ATP-dependent protease (Winsor et al. 2005) and 55% similarity to *E. coli* Lon protease, which has been shown to bind to TG-rich DNA promoter elements (Fu et al. 1997). PA3697, in turn, is annotated as a hypothetical protein with a possible metal-binding motif HEXXXH and nearby a perfectly conserved QEGLA motif, the function of which is yet unknown (Winsor et al. 2005). Protein homologs of PA3697 can be found only in other *Pseudomonas* species, of which PP1499 of *P. putida* shows 85% similarity with PA3697.

Characterization of PA0779 and PA3697 mutant strains of *P. aeruginosa*

To characterize PA0779 and PA3697 mutants, we analyzed their twitching and swimming, motilities, which both are implicated in the *P. aeruginosa* biofilm formation (Ramsey and Whiteley 2004), in addition to swarming motility. We did not detect any differences in the motilities of PA0779 and PA3697 mutants relative to PAO1. In addition, growth properties of the mutant strains versus PAO1 were undistinguishable under the conditions studied in this work, and both mutants readily formed biofilms (data not shown). Thus, PA0779 and PA3697 mutants do not show clearly distinguishable phenotypes in the tested conditions that would indicate their role or function in *P. aeruginosa*.

Transcriptional P_{fhp} and P_{fhpR} activities in *P. aeruginosa*

Recently, FhpR has been shown to upregulate *fhp* expression upon nitrosative stress (Arai et al. 2005). However, the in vitro binding studies with *fhp–fhpR* intergenic region suggest the involvement not only of FhpR, but also of PA0779 and PA3697 in the transcriptional regulation of either or both P_{fhp} and P_{fhpR} promoters. To assess the possible effects of PA0779 and PA3697 on P_{fhp} and P_{fhpR}, *P. aeruginosa* reporter strains carrying chromosomal integrants of the P_{fhp}/P_{fhpR} region fused with the *lacZ* reporter gene in both promoter orientations (P_{fhp}-*lacZ* and P_{fhpR}-*lacZ*) were constructed in PAO1 and strains deficient in either PA0779 or PA3697. The mutants were obtained from the mini-Tn5-*luxCDABE* mutant library (Lewenza et al. 2005). The mini-Tn5 derivative contains a promoterless *luxCDABE* operon and a tetracycline resistance cassette, which are flanked by the transposase recognition sequences (Winson et al. 1998). This transposon results in a knockout mutation when inserted into a gene, and in addition, it can lead to the production of a transcriptional *luxCDABE* fusion. However, the mutants used in this study were non-transcriptional fusions of the transposon within the ORFs of PA0779 and PA3697. As the gene encoding for PA0779 does not reside in an operon, and PA3697 is the last gene in a predicted operon, no polar effect should be present in the

used mutant strains. The transcriptional activities of both P_{fhp}-*lacZ* and P_{fhpR}-*lacZ* were measured from cultures grown under aerobic and low-oxygen conditions upon addition of SNP, NO_3^- and NO_2^-, which have previously been shown to induce P_{fhp} (Arai et al. 2005). Additionally, we studied the promoter activities upon exposure to $ONOO^-$, which is a strong nitration, but also an oxidation agent (Hughes 1999), formed during the host immune response. $ONOO^-$ is also suggested as a probable intermediate when oxygenated flavoHbs react with NO (Kim et al. 1999). All cultivations were performed in 96-deep well plates, which have been shown not to differ from growth in shake flasks in terms of mixing and oxygen transfer (Duetz et al. 2000; Minas et al. 2000). The use of 96-deep well plates enables cultivations of all the reporter strains at the same time in various conditions with minimal difference in handling and analysis.

The effect of PA0779 and PA3697 on P_{fhp} and P_{fhpR} activity under aerobic conditions

In PAO1, P_{fhp} activity was induced approximately 60-fold upon addition of SNP under aerobic conditions. Exposure to NO_3^-, NO_2^- and $ONOO^-$ induced P_{fhp} to much less extent, on the average 7- to 11-fold relative to uninduced control (Fig. 2a). This is in good agreement with the previously reported aerobic transcriptional activity of P_{fhp} (Arai et al. 2005). In PA0779 or PA3697 mutant strains, there was no P_{fhp} activity upon addition of NO_3^-, NO_2^- or in the untreated control under aerobic conditions. Only addition of SNP was able to induce P_{fhp} activity, which was 50 and 80% of the activity in PAO1 in the PA0779 and PA3697 mutants, respectively (Fig. 2a). In PAO1, P_{fhpR} activity showed similar induction pattern as P_{fhp} under aerobic conditions, being induced 77-fold with SNP, and only six to eightfold upon addition of NO_3^-, NO_2^- and $ONOO^-$ relative to the untreated control (Fig. 2b). In both mutant strains, SNP induced P_{fhpR} activity was 50% of P_{fhpR} activity in PAO1, showing that PA0779 and PA3697 are also needed for the full SNP induced P_{fhpR} activity under aerobic conditions. NO_3^- and NO_2^- induced P_{fhpR} activities were approximately the same in both mutant strains, but they were slightly lower compared to respective values in PAO1. Surprisingly, $ONOO^-$ induced P_{fhpR} activity was completely abolished in PA0779 mutant (Fig. 2b).

The effect of PA0779 and PA3697 in P_{fhp} and P_{fhpR} activity under low-oxygen conditions

During low-oxygen conditions there was a basal P_{fhp} activity in the untreated PAO1 and P_{fhp} activity was similar with the previous results obtained for P_{fhp} (Arai et al. 2005) when *P. aeruginosa* was treated either with SNP, NO_3^- or

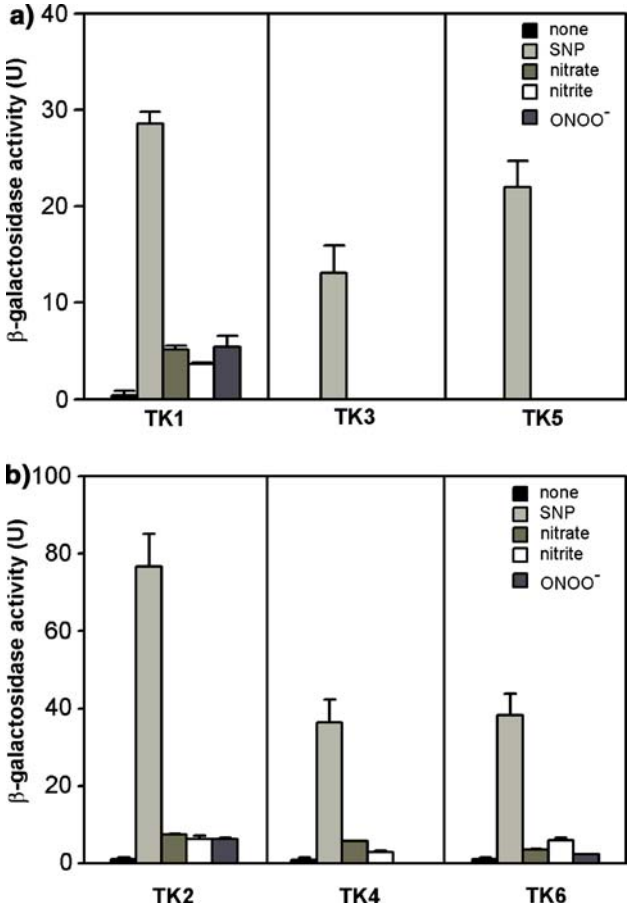

Fig. 2 Transcription of **a** P_{fhp} and **b** P_{fhpR} in PAO1 (TK1 and TK2), PA0779 mutant (TK3 and TK4) and PA3697 mutant (TK5 and TK6) under aerobic conditions. Cultivations were supplemented with sodium nitroprusside (SNP; 3 mM; *light grey bars*), nitrate (NO_3^-; 80 mM; *middle grey bars*), nitrite (NO_2^-; 20 mM; *white bars*) and peroxynitrite ($ONOO^-$; 1 mM; *dark grey bars*) 3.5 h after inoculation. Promoter activity was measured as β-galactosidase activity, which represents the promoter activity at 0.5 h after supplementation. Mean values and standard deviations of four separate cultivations are shown

NO_2^- under low oxygen conditions (Fig. 3a). SNP induced P_{fhp} activity was at the same level as under aerobic conditions, but NO_3^-, NO_2^- induced P_{fhp} activity was 6- and 5-fold higher, respectively, compared to the aerobic cultivation. The differences of P_{fhp} induction pattern during aerobic and low-oxygen conditions have been hypothesized to result from low expression levels of nitrate and nitrite reductases under the aerobic conditions limiting the formation of NO from nitrate and nitrite (Arai et al. 2005). P_{fhp} activity was not affected by the addition of $ONOO^-$ relative to untreated control under low-oxygen conditions. $ONOO^-$ is formed when NO reacts with Fe(III) hemes with bound superoxide and is the intermediate when a flavoHb detoxifies NO to NO_3^- aerobically. Thus, $ONOO^-$ cannot itself cause induction of P_{fhp} activity. Similarly as under aerobic conditions, in both PA0779 and PA3697 mutant strains, NO_3^- and NO_2^- induced P_{fhp} activity was completely abolished. In

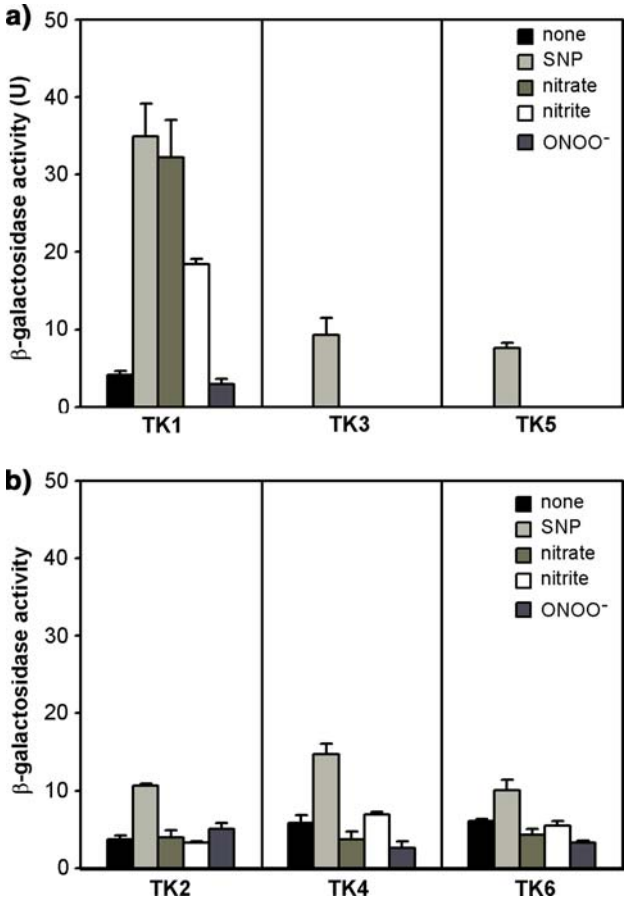

Fig. 3 Transcription of **a** P_{fhp} and **b** P_{fhpR} in PAO1 (TK1 and TK2), PA0779 mutant (TK3 and TK4) and PA3697 mutant (TK5 and TK6) under low-oxygen conditions. Cultivations were supplemented with sodium nitroprusside (SNP; 3 mM; *light grey bars*), nitrate (NO_3^-; 80 mM; *middle grey bars*), nitrite (NO_2^-; 20 mM; *white bars*) and peroxynitrite ($ONOO^-$; 1 mM; *dark grey bars*) at the point of inoculation. Promoter activity was measured as *β*-galactosidase activity, which represents the promoter activity 16 h after inoculation. Mean values and standard deviations of four separate cultivations are shown

addition, there was no basal level of P_{fhp} activity in the untreated control, which was present in PAO1. However, addition of SNP induced P_{fhp} in both mutants, but promoter activity was only 30% (PA0779 mutant) and 20% (PA3697 mutant) relative to P_{fhp} activity in PAO1 (Fig. 3a).

Interestingly, SNP induced P_{fhpR} activity was much lower than under aerobic conditions. In PAO1, SNP induced P_{fhpR} activity was only 2.9-fold higher relative to the untreated control. Addition of NO_3^- and NO_2^- did not cause any change in P_{fhpR} activity relative to the untreated control and P_{fhpR} activity was only half of the respective promoter activity values under aerobic conditions. Similar with the results obtained under aerobic conditions, neither PA0779 nor PA3697 had any significant effect on P_{fhpR} under low-oxygen conditions (Fig. 3b).

Effect of PA0779 and PA3697 on the activities of denitrification enzymes

Since the generation of NO from NO_3^- and NO_2^- is affected by NAR, NIR, and NOR, their enzymatic activities in anaerobically grown cells of the wild-type and PA0779 and PA3697 mutant strains were determined (Fig. 4). While no significant difference was observed in the NAR activity, the NIR and NOR activities of PA0779 and PA3697 mutant strains were significantly lower than those of the wild-type. In both mutants, the NIR and NOR activities were reduced to approximately 30 and 50%, respectively, of the activity in PAO1. However, no difference was found among the strains in the transcriptional activities of the *nirS* or *norC* promoters (data not shown). Thus, disruption of PA0779 or PA3697 seems to affect the post-translational activation of NIR and NOR. Moreover, the anaerobic growth on NO_3^- of both mutant strains was similar to that of the wild-type, displaying no differences in lag phase, during exponential growth and in stationary phase (Fig. 5). This suggests that the residual in vivo NIR and NOR activities are sufficient to maintain growth at an identical level as observed in wt PAO1 when using NO_3^- as an electron acceptor.

Discussion

Recently, it was shown that P_{fhp} is a σ^{54}-dependent promoter. FhpR is the cognate σ^{54}-dependent activator of P_{fhp} and it belongs to the NO sensing NorR-type prokaryotic

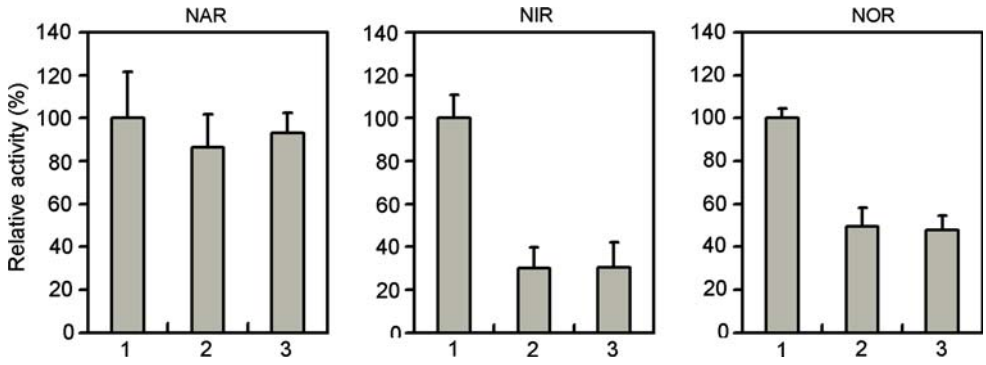

Fig. 4 Relative in vivo enzymatic activities of nitrate reductase (NAR), nitrite reductase (NIR) and nitric oxide reductase (NOR) in anaerobically grown cells of *P. aeruginosa* PAO1 wild-type (*1*), PA0779 mutant (*2*), and PA3697 mutant (*3*) strains. Mean values and standard deviations of more than three separate cultivations are shown

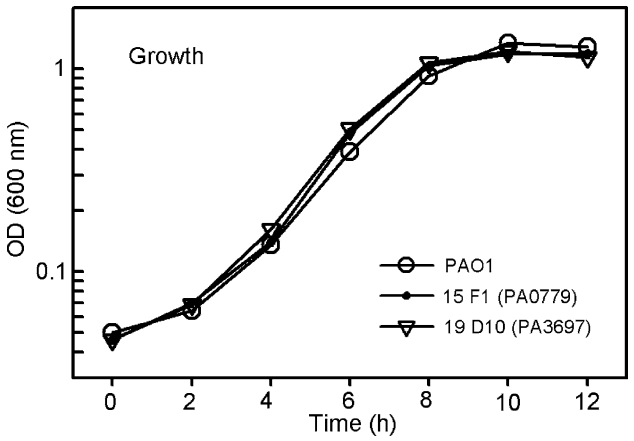

Fig. 5 Anaerobic growth of *P. aeruginosa* PAO1 wild-type (*open circle*), PA0779 mutant (*dot*), and PA3697 mutant (*open triangle*) strains in LB medium supplemented with 20 mM $NaNO_3$

enhancer binding proteins (Arai et al. 2005). In this study, biotinylated *fhp–fhpR* intergenic region DNA coupled to streptavidin coated magnetic beads was used to isolate proteins, which possibly have a role in the regulation of P_{fhp}/P_{fhpR}. Two previously uncharacterized proteins PA0779 (a probable Lon protease) and PA3697 were isolated and identified in addition to FhpR.

Closest homologs of PA0779 can be found in other *Pseudomonas* strains, in which they are annotated as Lon proteases or as in *P. entomophila* L48 as a DNA-binding ATP dependent protease (http://v2.pseudomonas.com). Lon proteases are tetrameric proteins conserved throughout prokaryotes, and they are typically activated by binding of ATP, DNA and target protein(s). Overall, Lon proteases have been shown to have various physiological and biochemical functions. For example, *B. subtilis* sporulation (Schmidt et al. 1994), and the switch of *Vibrio parahaemolyticus* between swarmer and planktonic cell types (Stewart et al. 1997) are regulated by Lon proteases. Lon proteases also affect capsule and exopolysaccharide production in bacteria (Gottesman et al. 1985; Eastgate et al. 1995). Interestingly in *P. aeruginosa*, there is also another Lon protease (PA1803), which has been recently shown to be essential for *P. aeruginosa* biofilm formation in addition to swimming, swarming, and twitching motilities (Marr et al. 2007). However, in our experiment the PA0779 mutant did not differ in its motility from PAO1. Therefore, it is clear that these two Lon proteases have different roles in this bacterium.

PA3697 is a protein, with so far no reported function or activity. The closest homologs of PA3697 can also be encountered in other *Pseudomonas* strains, but none of them have been characterized yet. However, proteins containing the QEGLA amino acid motif are also encountered in proteobacteria such as *Agrobacterium tumefaciens* and several species of *Vibrio* (Peterson et al. 2001). The SDS-PAGE gel band of PA3697 contained much more protein compared to the other protein bands (Fig. 1). This could indicate that PA3697 was isolated as an oligomer, and it has high affinity towards DNA or any of the other proteins involved in the transcription of P_{fhp}/P_{fhpR}.

The physiological role of Fhp in *P. aeruginosa* has been suggested to be detoxification of NO under aerobic conditions. FhpR activates the transcription of P_{fhp} in response to NO by binding to its three binding sites within the divergent P_{fhp}/P_{fhpR} promoter (Arai et al. 2005). Similarly, NorR activates the transcription of *norVW* in *E. coli*. Interestingly, both PA0779 and PA3697 are needed for the full NO-induced P_{fhp} activity under both aeration conditions, showing that they are part of the transcriptional machinery of P_{fhp} in addition to FhpR. Due to the divergent nature of the P_{fhp}/P_{fhpR} we also studied the transcriptional activity and regulation of P_{fhpR}. The *norR* promoter of *E. coli* is constitutive, and it has been found to be subject to negative autoregulation (Hutchings et al. 2002), which possibly results from the overlapping positions of the promoter elements and NorR boxes within the *norR–norVW* intergenic region (Tucker et al. 2006). According to the results of this study, *P. aeruginosa* P_{fhpR} is not constitutively active under aerobic conditions. High induction of P_{fhpR} activity is achieved with NO, and PA0779 and PA3697 are needed also for the full NO-induced P_{fhpR} activity. Neither addition of NO_3^- nor NO_2^- induces P_{fhpR} activity significantly under aerobic or low-oxygen conditions.

In addition to being induced significantly with NO under aerobic conditions, transcription of P_{fhp} was also highly induced with NO_3^-, NO_2^-, and NO under oxygen limiting conditions (Fig. 3a). Most interestingly, both PA0779 and PA3697 were shown to be essential for the transcriptional activity of P_{fhp} upon addition of NO_3^- and NO_2^- regardless of the oxygen level. Under low-oxygen or anoxic conditions, NO is formed endogenously through denitrification, and under such conditions high P_{fhp} activity was suggested to be induced NO-dependently via FhpR (Arai et al. 2005). However, according to the results obtained in this study, induction of P_{fhpR} by NO under low-oxygen conditions is very low compared to the induction under aerobic conditions. Thus, during denitrification, FhpR is not solely responsible for activation of P_{fhp}. Instead, PA0779 and PA3697 are essential for the transcriptional activity of P_{fhp} under such conditions. Furthermore, PA0779 and PA3697 were also found to have an effect on the post-translational regulation of NIR and NOR, but not on the transcriptional activities of *nirS* or *norC* promoters. Whenever enzymatic activity was reduced in the mutant strains versus PAO1, no changes were observed on the transcriptional level. However, despite their reduced NIR and NOR activities, both mutant strains grew indistinguishably from the wt PAO1

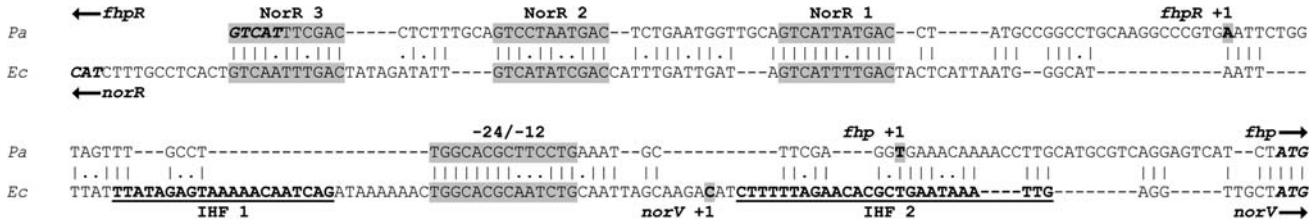

Fig. 6 Alignment of *P. aeruginosa fhp–fhpR* (*top*) and *E. coli norV-norR* (*bottom*) intergenic regions. Identical nucleotides are indicated by a *vertical line* and unidentical ones with a *dot*. NorR consensus binding sites (NorR 1–3) are highlighted in *grey*, binding sites for integration host factor (IHF) are in *bold* and *underlined* as well as the binding site for σ^{54}-RNA polymerase (−12/−24). Transcription start sites (+1) are in *bold* and highlighted in *grey*. DNA sequences belonging to the structural genes are marked in *italics* and indicated with arrows

under anaerobic conditions using NO_3^- as the sole electron acceptor. The low NIR activity in the PA0779 and PA3697 mutants under anaerobic and low-oxygen conditions might cause a low intracellular NO concentration, which in turn can have an additional effect on low P_{fhp} activity in these mutants in the presence of NO_3^- or NO_2^-.

The results of this study suggest that the mechanism of promoter activation by NorR-type proteins is different in *P. aeruginosa* and *E. coli*. In fact, alignment of the *E. coli* P_{norVW}/P_{norR} and *P. aeruginosa* P_{fhp}/P_{fhpR} promoter regions shows that the transcription start site of P_{fhpR} is not overlapping with the predicted NorR boxes (Fig. 6) as is the case for P_{norR} in *E. coli* (Tucker et al. 2005). In addition, upstream and downstream regions of the −12/−24 motif for σ^{54}-RNA polymerase binding are very different in P_{fhp}/P_{fhpR} and P_{norVW}/P_{norR}. Especially, P_{fhp}/P_{fhpR} does not contain the two integration host factor (IHF) binding sites, which are present in the P_{norVW}/P_{norR} (Tucker et al. 2005). As a result, these differences are not surprising as Fhp detoxifies NO under aerobic conditions, whereas in *E. coli* NorVW detoxifies NO under anaerobic conditions.

Conclusions

It has been shown previously that flavoHbs from various bacteria are conserved in sequence and their role and function are very similar (Bollinger et al. 2001). However, the regulation mechanisms of *flavoHbs* from different bacteria seem to be very complex. The type of proteins involved in the *flavoHb* regulation is species dependent, but similarities also exist. *P. aeruginosa* Fhp is conserved among different isolates such as PAO1, PA 14 (Winsor et al. 2005) and several clinical isolates (T. Koskenkorva, unpublished results), and the flavoHb proteins of *P. putida*, *P. fluorescens*, *P. syringae* and *P. entomophila* show also high degree of similarity (Winsor et al. 2005). Interestingly, homologs of PA0779 and PA3697 proteins seem to be encountered only in the above-mentioned Pseudomonads, which also have a similar chromosomal *flavoHb* gene and NorR-type regulator configuration as is observed in *P. aeruginosa*. We have also identified −12/−24 motifs and putative NorR boxes within the *flavoHb* upstream regions of *P. putida*, *P. fluorescens*, and *P. syringae* (T. Koskenkorva, unpublished results). Thus, it is possible that also in these *Pseudomonas* species the transcriptional regulation of *flavoHb* genes is similar and may require homologs of FhpR, PA0779 and PA3697 proteins. Also, the involvement of PA0779 and PA3697 in controlling other factors than Fhp/FhpR system cannot be ruled out.

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